

LABELLED PEPTIDES HAVING AFFINITY FOR A
PHOSPHOLIPID AND USES

DESCRIPTION

5

TECHNICAL FIELD

The present invention relates to a family of peptides labelled with fluorine-18 which have enhanced affinities for phospholipids, and to their uses.

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In general, the peptides of the present invention are useful for the specific recognition of lipid molecules. They can be used for engineering and creating compounds recognizing and sequestering lipids, in particular negatively charged lipids, such as phosphatidylserines, phosphatidic and lysophosphatidic acids, phosphatidylglycerols, cardiolipins and sphingosine-1-phosphates.

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The abovementioned lipids play an important role, in particular in cell signalling and may be present at the outer surface of cell membranes and/or circulate in the blood stream following a wide variety of pathological events.

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Various cellular events result in the appearance of negatively charged lipids and in particular of phosphatidylserines (PS) at the outer surface of cells; these events can result either from a fortuitous or pathological impairment of the cell, or from a programmed cellular event such as cell death or apoptosis. The appearance of PS at the outer surface of cells therefore constitutes an important "primary message" indicating the existence of a dysfunction. In the case of the blood clotting process, the mechanism is well described: the impairment of the endothelial cells of the blood vessels, either for accidental reasons, or for more complex pathological reasons,

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causes the appearance of this PS message at the outer surface of the cells in contact with the blood stream. This message is immediately recognized by certain circulating proteins which then trigger a cascade of events resulting in the well-known phenomenon of blood clotting.

The invention exploits the property of the labelled peptides, which it provides, to bind, in the presence or absence of calcium, to lipids and in particular to those which are negatively charged, for the development of compounds which can be used as research and diagnostic tools in the field of the recognition of lipid effectors and of the detection of apoptosis, of blood clotting disorders, of septic shock and of acute inflammatory pathologies in particular.

The labelled peptides of the invention are coupled to a radioactive halogen, a positron emitter, which is fluorine ¹⁸F. With these labelled peptides, it is therefore possible, for example to detect apoptotic cells and to recognize negatively charged membrane microdomains.

They can be used for "in vitro" detection of pathologies involving the appearance of centres exposing negatively charged lipids at the surface of the cells and/or the release of microvesicles into the blood.

The labelled peptides of the present invention may also be used for the in vivo detection and the imaging of apoptotic foci, of thrombotic regions, and in general of centres exposing negatively charged lipids at the surface of cells and/or the release of microvesicles into the blood, for example by means of scintigraphic images, acquired by positron emission tomography (PET).

Other applications will also appear to persons skilled in the art on reading the description which follows.

STATE OF THE ART

5 A family of proteins, called annexins, have been described in the prior art as presenting a reversible functional anchor to the cell membrane, regulated by the calcium concentration and the presence of anionic phospholipids. Annexins constitute a family of proteins
10 expressed in a wide variety of tissues, both in animals and in plants. It appears that they are not expressed either in bacteria or in yeast.

The structure of annexins comprises four domains of
15 about 70 amino acids, or residues, with a very slight sequence homology, but with practically identical topology.

In the document WO 92/19279, J. TAIT describes
20 conjugates having affinity for phospholipids. It describes in particular the use of an annexin, in particular of annexin V, to manufacture an active conjugate which can be used as thrombolytic agent.

25 Unfortunately, the compound described in this document and prepared from whole annexin by a method of genetic recombination has many disadvantages which are in particular a low yield and a high cost of manufacture. The major disadvantages are especially the production
30 of a fragile conjugate because of its complex topology resulting in irreversible unfolding. In addition, these molecules exhibit a major toxicity for the kidney and the heart.

35 The present inventors have described, in application WO-A-00/20453, a first family of peptides overcoming the abovementioned disadvantages and exhibiting affinity for phospholipids and enhanced stability.

Moreover, it is known that for use in research and diagnosis, macromolecules, such as proteins or peptides, can be coupled to a labelling molecule allowing their detection, this labelling molecule may be for example a fluorescent molecule, gold particles, a paramagnetic compound or a molecule bearing a radioelement.

Proteins have been radioactively labelled with radioisotopes, iodine and various radioisotopes of metals, such as technetium, indium and gallium. More recently, proteins have been labelled with fluorine-18.

For example, peptides coupled to radioelements, such as fluorine, allow "in vivo" detection of the localization of thrombotic regions during all sorts of stroke, in particular of apoptotic and inflammatory foci, using imaging systems.

Thus, radioactive atoms which emit positrons having a short life span, and especially ^{18}F , can in particular be detected by positron emission tomography (PET) apparatus.

Radioactive labelling with fluorine-18 poses, in particular because of the very short life span of fluorine-18 (close to 109.8 minutes), specific problems which are such that labelling with fluorine-18 is basically different from that with other halogens, such as iodine.

The abovementioned labelling may be carried out by any of the conventional techniques of organic chemistry known to persons skilled in the art, and by the synthesis of protein and peptide markers bearing one or more radioactive atoms with a short life span, in particular ^{18}F . This marker generally consists, on the

one hand, of a part capable of receiving, for example,
an atom of ^{18}F and, on the other hand, of a part
containing any conventional functional group for
linking to the macromolecule, for example to the
5 protein.

These markers must satisfy the requirement for rapid
and easy synthesis, because due to the short life span
of radioisotopes such as ^{18}F , the duration of synthesis
10 should generally not exceed a few hours.

In addition, this synthesis, because of the high
radioactivity of the compounds used, must be capable of
being carried out by automated means.

15 Thus, the methods for labelling proteins or peptides
with fluorine-18 involve markers also called labelled
"conjugates" or "synthons", which are classified into
three main families, depending on whether they react
20 with the amine groups, the sulphhydryl groups, or the
carbohydrate groups of the macromolecules, such as
proteins and peptides.

Among the compounds or conjugates reacting with amino
25 groups, there may be mentioned imidates, such as 3-
[^{18}F]fluoro-5-nitrobenzoimidate, which react, for
example, with the $\epsilon\text{-NH}_2$ group of lysine in order to bind
to a protein; activated esters, such as N-succinimidyl-
[^{18}F]fluorobenzoate; carboxylic acids, such as N-(4-
30 [^{18}F]fluorobenzoic) acid; aldehydes, such as 4-
[^{18}F]pentafluorobenzaldehyde and isothiocyanates, such
as 4-([^{18}F]fluoromethylphenylisothiocyanate).

Activated halides, such as (4-[^{18}F]fluorophenacyl)
35 bromide, react with the amino groups, such as the $\epsilon\text{-NH}_2$
group of lysine and the -SH group of cysteine.

Amines, such as 1-(4-
([¹⁸F]fluoromethyl)benzoyl)aminobutane-4-amine, react
with the CO₂H groups, for example of glutamic acid or
of aspartic acid or with the CHO groups of
5 glycoproteins.

Nitrenes with photochemical active centres, such as
azidophenacyl [¹⁸F]fluoride, also react with the amino
groups, for example the ε-NH₂ group of lysine.

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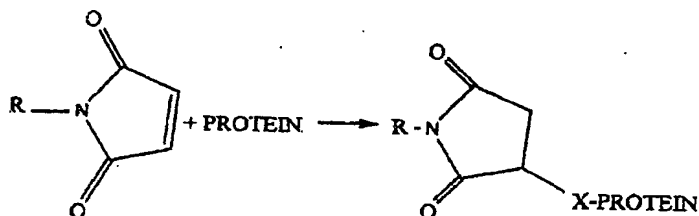
The most effective and most widely described method for
labelling proteins and peptides is that which uses
activated acids, but it is also the method which
exhibits the greatest nonspecificity because all the
15 nucleophilic sites of the amino acids of the proteins
or peptides will react with the labelled marker,
conjugate or synthon.

Two methods which are more specific for labelling
20 peptides and nucleotides exhibit good specificity
toward the sulphur atoms, for example, of cysteine for
peptides and for a phosphorothioate functional group
for nucleotides.

25 They include, first of all, methods using haloacetamide
"synthons" which, although satisfactory, have the
disadvantage of being very slow and therefore not very
suitable for ¹⁸F, because of its life span.

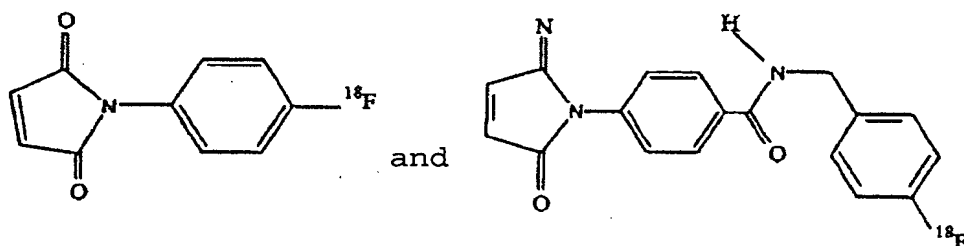
30 They then include methods using activated maleimides
which can bind to the SH groups with a very good
specificity because the reaction is very slow in
relation, for example, to the ε-NH₂ sites of lysine.

35 The reaction scheme involving the maleimido group is
the following, in the case of a protein:



in which X represents -S-.

- 5 For any labelling, regardless of the type, molecules comprising a maleimide radical are currently considered as being the best, as regards their reactivity with macromolecules, such as peptides or proteins.
- 10 The document by SHIUE C.-Y. et al., J. Label Compounds Radiopharm 26: 278-280 (1988), describes the compounds:



- 15 The first of these compounds is not easy to label with fluorine-18 at a high specific activity.

Indeed, only fluorine F_2 would allow easy labelling of the "iodine type" and it happens to be the case precisely that F_2 is generally a product with a low specific activity.

In particular F_2 is not suitable for the manufacture of so-called "radiotracer" compounds which are preferably aimed at according to the invention quite simply because the injected mass of labelled molecule becomes too large and in that case the basic principle guiding

this "tracer", namely the extremely easy occupation (for example less than 5%) of the receptor sites, is not satisfied.

5 In addition, the synthesis of the first of these compounds is difficult; it is indeed carried out in four stages requiring a long period with very low yields, and relatively complex chemical conversions. This method cannot therefore be easily automated.

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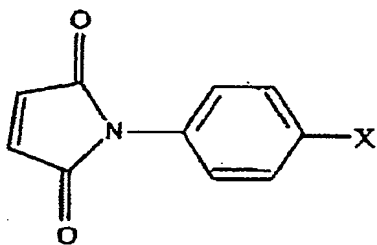
The second of the compounds cited in the SHIUE et al. document contains an amide chain which is not chemically very strong and which is easily cleaved or broken in vivo.

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Its use for diagnostic applications cannot therefore be envisaged. In addition, the synthesis of this second compound comprises three stages and the final yield is low, for example close to 10% ("EOB" "End of Bombardment").

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The document US-A-4 735 792 relates to molecules of formula:



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in which X is a radioactive halogen chosen from bromine-75, bromine-76, bromine-82, iodine-123, iodine-125, iodine-131 and fluorine-18.

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However, only the molecule labelled with iodine-125 is effectively prepared.

The preparation of a molecule labelled with fluorine-18 is not mentioned or evoked, and the remarks already made above in the case of the first compound of the
5 SHIUE et al., document also apply in the case of the document US-A-4 735 792.

Persons skilled in the art, on reading this document, possess no information allowing them to specifically
10 prepare a compound labelled with fluorine-18 and if they envisage doing it, they would use F₂ and would thus arrive at a compound with a low specific activity, which is unusable in "PET" imaging.

15 It can be considered additionally that the chemistry used to manufacture the fluorinated compound of the document US-A-4 735 792 is a complex and long chemistry.

20 DISCLOSURE OF THE INVENTION

The aim of the present invention is precisely to provide a novel family of peptides, labelled with a radioactive halogen which is fluorine ¹⁸F using a novel
labelling compound, the peptide having affinity for
25 lipids, in particular for phospholipids, more specific and further improved compared with the prior art products, and the labelling compound having, inter alia, high reactivity, high selectivity in particular towards sulphur atoms such as those of the thiol
30 functional groups of cysteines, and a good specific activity and it being possible for the said labelling compound in addition to be manufactured by a method which is simple, reliable, easily automatable, rapid and of short duration.

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The peptides of the invention have in addition the advantages of being chemically more stable than the prior art compounds and of being able to be

manufactured reproducibly, with a high yield and a very low cost of production compared with the prior art compounds.

5 Fluorine-18 (^{18}F) is a positron emitter which allows detection, by means of the labelled peptides of the present invention, of negatively charged lipids in any region of the body by positron (PET) cameras. This coupling of the peptides of the present invention to ^{18}F
10 makes it possible for example to detect, with a resolution better than the millimetre range, the presence of cells exhibiting phosphatidylserine (PS), present at the outer surface of the cells involved in physiopathological processes such as programmed cell
15 death, apoptosis, blood clotting, inflammatory reaction in vivo in any living being. It also allows such a detection in vitro in laboratory tests.

These labelled peptides of the present invention also
20 make it possible to precisely quantify for example the number of cells having phosphatidylserine.

The peptides of the present invention are characterized in that they comprise the following peptide sequence
25 (PI):

$\text{J}^1\text{-J}^2\text{-J}^3\text{-J}^4\text{-J}^5\text{-J}^6\text{-Z}^7\text{-U}^8\text{-J}^9\text{-J}^{10}\text{-U}^{11}\text{-Arg-J}^{13}\text{-J}^{14}\text{-U}^{15}\text{-Lys-}$
 $\text{Gly-X}^{18}\text{-Gly-Thr-J}^{21}\text{-Glu-J}^{23}\text{-J}^{24}\text{-U}^{25}\text{-J}^{26}\text{-J}^{27}\text{-J}^{28}\text{-U}^{29}\text{-J}^{30}\text{-J}^{31}\text{-}$
 $\text{Arg-J}^{33}\text{-J}^{34}\text{-J}^{35}\text{-J}^{36}\text{-B}^{37}\text{-J}^{38}\text{-J}^{39}\text{-U}^{40}\text{-J}^{41}\text{-J}^{42}\text{-J}^{43}\text{-U}^{44}\text{-J}^{45}\text{-J}^{46}\text{-J}^{47}\text{-}$
 $\text{J}^{48}\text{-J}^{49}\text{-Arg-J}^{51}\text{-U}^{52}\text{-J}^{53}\text{-J}^{54}\text{-Asp-U}^{56}\text{-Lys-Ser-Z}^{59}\text{-Leu-J}^{61}\text{-J}^{62}\text{-}$
 $\text{J}^{63}\text{-J}^{64}\text{-Z}^{65}\text{-J}^{66}\text{-J}^{67}\text{-U}^{68}\text{-J}^{69}\text{-J}^{70}\text{-J}^{71}\text{-U}^{72}\text{-J}^{73}\text{-J}^{74}\text{-J}^{75}$ (PI)

in which J, Z, U, X and B represent amino acids such
30 that:

- the amino acids J are chosen independently of each other from natural amino acids, or derivatives thereof, in such a manner that at least 50% of

them are polar residues chosen from Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Lys, Orn, Pro, Ser, Thr and Tyr,

5 the amino acids U are chosen from Ala, Cys, Gly, Ile, Leu, Met, Phe, Trp, Tyr and Val,

- the amino acid X^{18} is chosen independently of the other amino acids of the sequence from Ala, Asn, Cys, Gln, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val,

10 - the amino acid B^{37} is chosen independently of the other amino acids of the sequence from Arg, Ala, Cys, Gly, Ile, Leu, Met, Phe, Trp, Tyr and Val,

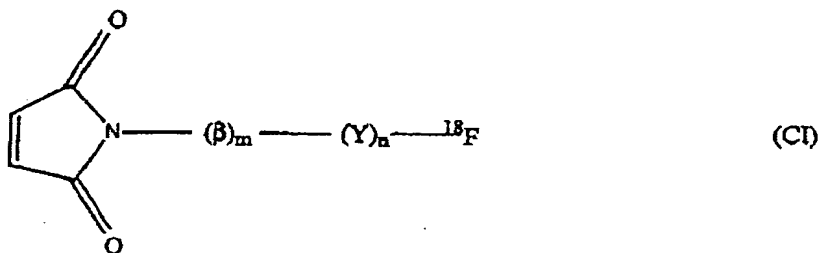
- the amino acid Z^7 is chosen independently of the other amino acids of the sequence from Asp and Glu,

15 the amino acids Z^{59} and Z^{65} are chosen independently from Glu, Asp, Lys and Arg,

the superscripts of J, Z, U, X and B representing the positions of these amino acids in the said sequence.

20 According to the invention, these peptides of the present invention, as defined above, are labelled directly or indirectly with a labelling compound of the present invention of the following general formula

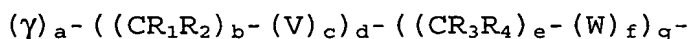
25 (CI):



in which:

30 - m represents an integer from 0 to 10, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10;

- n represents an integer from 0 to 10, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10;
- Y represents a group chosen from alkyl groups, monocyclic or bicyclic heterocyclic groups chosen from imidazolyl, pyrazolyl, benzimidazolyl, pyridinyl, piridazinyl, pyrimidinyl, pyrazinyl, triazinyl, quinolinyl, isoquinolinyl, cinnolinyl, quinazolinyl, quinoxalinyl and purinyl groups, it being possible for Y to be optionally substituted with one or more substituents, each of these substituents being chosen independently from hydrogen, (nonradioactive) halogens, phenyl, C₁₋₆ alkyl, C₁₋₆ alkoxy, aryloxy, amino, mono- or di(C₁₋₆ alkyl)amino, mono- or di(aryl)amino, thio, C₁₋₆ alkylthio, arylthio, formyl, C₁₋₆ alkylcarbonyl, arylcarbonyl, carbonyl, C₁₋₆ alkoxycarbonyl, aryloxycarbonyl, C₁₋₆ alkylaminocarbonyl, arylaminocarbonyl and trifluoromethyl groups;
- β represents a radical of formula:



in which:

- a, b, c, d, e, f, g each independently represent an integer from 0 to 10, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9;
- γ, V and W each independently represent -NR₁-, -O-,

$$\begin{array}{c} \text{O} \\ | \\ \text{---N---} \end{array}$$
 , ethynyl, -CR₁=CR₂-, -(C=O)-, -(C=S)-, -C(=NR₁)-, -C(=O)O-, -(C=S)S-, -C(=NR₁)NR₂-, -CR₁R₂-, -CR₁OR₂-, -CR₁NR₂R₃-, where R₁, R₂, R₃ and R₄ are independently chosen from hydrogen, halogens, phenyl, C₁₋₆ alkyl, C₁₋₆ alkoxy, aryloxy, amino, mono- or di(C₁₋₆ alkyl)amino, mono- or di(aryl)amino, thio, C₁₋₆ alkylthio, arylthio, formyl, C₁₋₆ alkylcarbonyl, arylcarbonyl, carbonyl (C₁₋₆)alkoxycarbonyl, aryloxycarbonyl, C₁₋₆

alkylaminocarbonyl, arylaminocarbonyl and trifluoromethyl groups.

5 Generally, in the present description, halogen means fluorine, chlorine, bromine or iodine. C₁₋₆ alkyl corresponds to linear and branched chain saturated hydrocarbon radicals having from 1 to 6 carbon atoms, such as methyl, ethyl, propyl, butyl, pentyl and hexyl.

10 The attachment and the substitution of the heterocycles, aryl group, and the like, may be made in any position.

15 Likewise, the attachment of the ¹⁸F to Y or β may be made in any position, in particular to any position on a heterocycle.

The compounds according to the present invention are basically distinguishable from the prior art compounds because of their specific structure in which the part bearing the fluorine-18 atom consists, according to the invention, of a specific group Y which is in particular a pyridinyl group; the part for linking, coupling to the peptide consists, according to the invention, of a specific functional group, namely a maleimido functional group; and, finally, the part for binding to the peptide and the part bearing the fluorine-18 atom are linked according to the invention by a spacer chain or arm which is also specific, for example of the type comprising alkyl (generally from 2 to 6C), alkyl ether, phenylalkyl ethers, alkenyl, which are not fragile and are not susceptible to breakings "in vivo".

35 The expression direct labelling is understood to mean a direct coupling, without intermediate, such as a spacer arm, of the labelling compound (CI) with the peptide of the present invention, for example by means of a free -SH functional group of the peptide defined above; this

may be in particular the thiol functional group of a cysteine of the peptide.

5 This coupling of the labelling compound (CI) with the peptide can be carried out either on the sequence (PI) defined above, for example on cysteine residues localized at the surface of the protein, but in a manner which is not disruptive for the functional groups for binding calcium and phospholipids, or on a
10 portion of the peptide other than that of the said sequence (PI). The coupling occurs through the maleimide functional group of the compound (CI).

15 More precisely, the said coupling is achieved by the reaction of the double bond of the maleimido group of the compound according to the invention with specifically an -SH (thiol) functional group of a cysteine forming part of the peptide.

20 One of the advantages linked to the specific structure of the compounds according to the invention is to allow specific, or even exclusive, labelling of the cysteines, whereas most of the other "synthons" only allow nonspecific labelling of the lysines and of the
25 cysteines.

The selective, or even exclusive, labelling of the cysteines is due to the presence, in the labelling molecule of the invention, of a "dedicated" functional
30 group, namely the maleimido functional group, which is a dedicated functional group for the chemoselectivity towards the thiols of the cysteines.

The expression indirect labelling is understood to mean
35 the use of a spacer arm linked, on the one hand, to the labelling compound, and, on the other hand, to the peptide as defined above. This spacer arm may have the role of putting the marker and the peptide apart so

that no steric hindrance prevents the peptide from recognizing its target (negatively charged lipid). This spacer arm may be of an organic nature, for example an alkyl provided with a thiol group, or a peptide
5 sequence comprising a cysteine, for example $-(\text{Gly})_n\text{-Cys}$ where n is equal to or greater than 1.

It is evident that the coupling of the labelling compound with the peptide in accordance with the
10 present invention will be in any case such that it does not inhibit or inhibits in a manner which is not very disruptive the activity for the specific recognition of the negatively charged lipids by the peptide of the present invention.

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The above peptide sequence (PI) falls in space in order to adopt its tertiary conformation which is the active form of the peptide.

20 The amino acids 12, 15, 16, 17, 19, 20, 22, 50, 55, 57, 58, 59, 60 and 65 of the peptide (PI) of the present invention are amino acids, or residues, involved directly or indirectly in the binding to lipids, that is to say that they are involved either in the three-
25 dimensional structure of the peptide so that it adopts its active conformation for recognition, or in the site for recognition of the lipid.

The amino acids J are the surface amino acids or
30 residues of this peptide when it is in its folded and active conformation. These residues are arranged in space such that they are partially or completely exposed to the solvent. According to the present invention, these amino acids J may for example be
35 chosen independently of each other from all the natural amino acid residues Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, and Val and in such a manner that at least

50% of them are polar residues chosen from Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Lys, Orn, Pro, Ser and Thr. Examples are given in the appended sequence listing.

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The amino acids U are the core residues of this peptide. In the folded and active conformation of the peptide, they are arranged in space close to each other and not exposed to the solvent. They constitute the hydrophobic core of the protein. The compact assembly of the atoms of these residues plays a predominant role for the stability of the peptide in its active conformation. These residues may be chosen from the list of amino acids U described above. Various examples of combinations of core residues in the peptide sequence (PI) of the present invention are given in the table (1) below:

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Table 1

	U ⁸	U ¹¹	U ¹⁵	U ²⁵	U ²⁹	B ³⁷	U ⁴⁰	U ⁴⁴	U ⁵²	U ⁵⁶	U ⁶⁸	U ⁷²
Ex a)	Val	Leu	Met	Ile	Leu	Arg	Ile	Tyr	Leu	Leu	Val	Leu
Ex b)	Ala	Ile	Ile	Ile	Leu	Arg	Ile	Tyr	Leu	Leu	Ile	Leu
Ex c)	Ala	Ile	Ile	Ile	Leu	Arg	Ile	Tyr	Leu	Leu	Met	Val
Ex d)	Ala	Leu	Met	Leu	Leu	Arg	Ile	Tyr	Leu	Leu	Ile	Met
Ex e)	Ala	Leu	Met	Ile	Ile	Arg	Val	Tyr	Leu	Leu	Ile	Met
Ex f)	Ala	Leu	Met	Ile	Ile	Arg	Ile	Phe	Leu	Leu	Ile	Met
Ex g)	Ala	Leu	Met	Ile	Val	Arg	Ile	Phe	Leu	Leu	Ile	Phe
Ex h)	Val	Leu	Met	Ile	Leu	Arg	Ile	Phe	Leu	Leu	Ile	Met
Ex i)	Ala	Leu	Met	Ile	Leu	Arg	Ile	Phe	Leu	Leu	Ile	Met
Ex j)	Ala	Leu	Met	Ile	Leu	Arg	Ile	Tyr	Leu	Leu	Ala	Ala
Ex k)	Val	Leu	Met	Ile	Leu	Arg	Ile	Tyr	Leu	Leu	Val	Leu
Ex l)	Val	Leu	Met	Ile	Leu	Arg	Ile	Phe	Leu	Leu	Val	Leu

(Ex = exemple)

5 (Ex = Example)

The residue X¹⁸ has the role of maintaining the structure of the Gly-X-Gly loop in the active form of the peptide, in particular where the residues Z⁵⁹ and Z⁶⁵ are Glu, to modulate the hydrophobic and lipophilic character of this loop, and to optionally provide specific novel interactions with phospholipids. That is the case for example for the residues Asn, Cys, Ser, Thr, Trp and Tyr.

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The residues Z⁵⁹ and Z⁶⁵ may be advantageously lysine residues, which has the effect of replacing the calcium ion with the positively charged group -NH₃⁺ of the

lysine and of enhancing the affinity of the peptide for a negatively charged membrane.

5 The peptide (PI) of the present invention, in its active form, comprises three sites for binding to a calcium ion where the calcium ion complexed by this site constitutes one of the ligands of a negatively charged phospholipid. The first of these sites, called principal site, involves the residues 15, 18, 19 and 59
10 as calcium ligands. The second of these sites, called secondary site, involves the residues 20 and 22 as calcium ligands. The third of these sites, which is a secondary site of low affinity involves the residues 57, 60 and 65 as calcium ligands.

15 The residues which are involved overall in the binding to the phospholipids are the residues 12, 15, 16, 19, 20, 22, 50, 55, 57, 58, 59, 60 and 65. This list includes residues involved in the bindings of calcium,
20 the phospholipids being calcium ligands.

These residues may of course be replaced by residues playing the same role for the purpose of the same result in accordance with the present invention.

25 By way of example, according to the invention, the peptide of formula (PI) may be advantageously a peptide sequence chosen from the appended peptide sequences ID No. 1 to ID No. 10.

30 The sequence (PI) represents the peptides of the present invention in their shortest functional form. It is of course understood that this sequence may additionally comprise, linked to the N-terminal end and/or to the C-terminal end of the sequence (PI), one
35 or more amino acids, for example from 1 to 15 amino acids, in general from 1 to 10 amino acids. Most preferably, these additional amino acids do not modify

or only slightly modify the activity of the peptides, or else enhance it.

For example, a small sequence, called below
5 functionalization sequence may be useful in particular for attaching a marker to the peptide, for attaching a molecule for treating diseases to the peptide and/or for attaching the said peptide to a support. The length
10 of this functionalization sequence may be adapted according to its use. Of course, the latter will preferably not interfere with the activity of the peptide of the present invention. Persons skilled in the art will easily know how to adapt the length and the nature of this functionalization sequence according
15 to the use that will be made of a peptide of the present invention.

Thus, according to a first particular embodiment of the present invention, the peptides of the present
20 invention may contain, for example at the N-terminal end, a functionalization sequence of three amino acids. This functionalization sequence allows direct attachment of the labelling compound (CI) to the peptide. The peptides in accordance with this
25 embodiment may be defined by the following sequence (PII):

J^{-2} - J^{-1} - J^0 - J^1 - J^2 - J^3 - J^4 - J^5 - J^6 -Z⁷-U⁸- J^9 - J^{10} -U¹¹-Arg- J^{13} - J^{14} -U¹⁵-
Lys-Gly-X¹⁸-Gly-Thr- J^{21} -Glu- J^{23} - J^{24} -U²⁵- J^{26} - J^{27} - J^{28} -U²⁹- J^{30} -
 J^{31} -Arg- J^{33} - J^{34} - J^{35} - J^{36} -B³⁷- J^{38} - J^{39} -U⁴⁰- J^{41} - J^{42} - J^{43} -U⁴⁴- J^{45} - J^{46} -
 J^{47} - J^{48} - J^{49} -Arg- J^{51} -U⁵²- J^{53} - J^{54} -Asp-U⁵⁶-Lys-Ser-Z⁵⁹-Leu- J^{61} -
 J^{62} - J^{63} - J^{64} -Z⁶⁵- J^{66} - J^{67} -U⁶⁸- J^{69} - J^{70} - J^{71} -U⁷²- J^{73} - J^{74} - J^{75} (PII)

30 in which J, Z, U, X and B are as defined above.

For example, J^{-2} may be Gly, J^{-1} may be Ser or Cys and J^0 may be Cys, Thr, Pro, Ser or Gln, preferably J^0 is Cys.

This sequence $J^{-2}J^{-1}-J^0$ may be chosen for example from Gly-Ser-Cys-, and Gly-Cys-Ser-. Thus, for example, each of the sequences ID No. 1 to ID No. 10 mentioned above may contain according to choice each of the
5 abovementioned functional sequences. The sequence ID No. 12 of the appended sequence listing is only a nonlimiting example of a sequence (PII) according to the present invention containing at its N-terminal end a functional sequence of three amino acids.

10

According to a second particular embodiment of the present invention, the peptide sequence (PI) may contain, for example at their N-terminal end, a functionalization sequence of four amino acids
15 $J^{-3}-J^{-2}J^{-1}-J^0$ chosen from Gly-Ser-Gly-Cys-, Gly-Cys-Gly-Ser, and Gly-Cys-Gly-Cys. This functionalization sequence is useful for example for direct attachment of the labelling compound (CI) to the peptide. Thus, for example, each of the abovementioned
20 sequences ID No. 1 to ID No. 10 may contain according to choice each of the abovementioned functional sequences. The sequences ID No. 11 of the appended sequence listing (several sequences are grouped under a single name ID No. 11) are only nonlimiting examples of
25 sequences (PI) according to the present invention containing at its N-terminal end a functional sequence of four amino acids.

According to a third particular embodiment of the
30 present invention, the peptide sequences (PI) may contain, for example at their N-terminal end, a functionalization sequence of seven to eleven amino acids. This functionalization sequence is also useful for directly attaching the compound (CI) to the
35 peptide. This embodiment is set out below. Thus, for example, each of the sequences ID No. 1 to ID No. 10 mentioned above may contain according to choice each of the abovementioned functional sequences. It is also

possible to replace the sequence Gly-Ser-Gly-Cys of the sequences ID No. 11 to 14 by Gly-Bb1-Gly-Bb2, in which Bb1 and Bb2 are independently Cys or Ser. The sequences ID No. 13 and 14 of the appended sequence listing
5 (several sequences are grouped into one under the name ID No. 13 or 14) are merely nonlimiting examples of such peptides.

The peptides of the present invention have a sufficient
10 affinity for calcium and are capable of reversibly binding to lipid effectors, and in particular to those which are negatively charged, such as phosphatidylserines, phosphatidic acids, phosphatidylethanolamines, phosphatidylglycerols,
15 cardiolipins and phosphatidylinositolphosphates.

This is a family of peptides whose main property is to specifically recognize the appearance of lipid signals at the surface of cell membranes in relation to the
20 normal or pathological function of the tissues.

The peptides of the present invention may be synthesized by conventional methods of organic chemistry synthesis or of protein chemistry, and by
25 genetic recombination *in vivo* or *in vitro*, by genetic engineering, and the like.

The peptide according to the invention may be synthesized by solid-phase chemical synthesis of the
30 said peptide. This chemical synthesis may be carried out for example with an automatic peptide synthesizer of the Applied Biosystems, mod.433A type. It may be carried out for example by Fmoc chemistry which uses the fluorenylmethyloxycarbonyl group for the temporary
35 protection of the α -amino functional group of amino acids.

The technical elements for the implementation of this method of peptide synthesis are known to persons skilled in the art. They are described for example in the manual Solid-Phase Organic Synthesis by Kevin Burgess (Editor) Wiley-Interscience; ISBN: 0471318256; (February 2000).

The peptide of the invention may also be manufactured by genetic recombination *in vivo* for example by means of a method comprising the following steps:

- a) preparation of a cDNA comprising a basic sequence encoding the said peptide
- b) insertion of the said cDNA into an appropriate expression vector,
- c) transformation of an appropriate host cell with the said vector into which the cDNA has been inserted, for replication of the plasmid,
- d) manufacture of the said peptide by translation of the said cDNA in the said host cell, and
- e) recovery of the peptide synthesized.

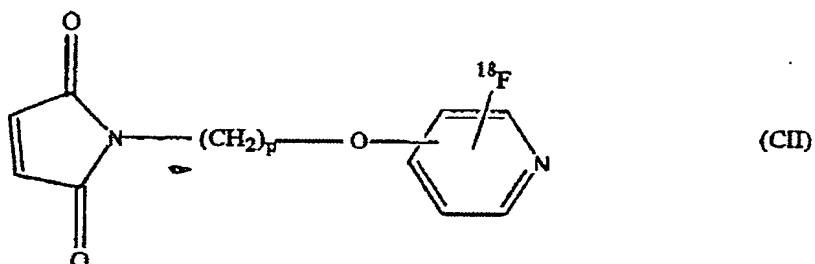
According to the invention, the appropriate expression vector and the host cell are chosen according to the usual techniques for genetic recombination. The vector may be any of the plasmids generally used in this technique, for example a plasmid such as the vector pGEX-2T. Likewise, the cell may be chosen according to the usual techniques; it may be for example *E. coli*.

When a genetic recombination technique *in vitro* is used, steps c) and d) of the above method are replaced respectively by steps c') for introducing the vector into which the cDNA has been inserted in a suitable reaction medium for replication of the plasmid, and d') for manufacture of the said peptide by translation of the said cDNA in the said suitable reaction medium. The document Jagus, R. and Beckler, G.S. (1998) Overview of eukaryotic *in vitro* translation and expression systems,

Current Protocols in Cell Biology 11.1.1-11.1.13., 1998 by John Wiley & Sons, Inc. describes methods *in vitro* which can be used in the present invention.

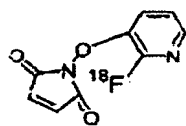
- 5 According to the invention, advantageously, in the above labelling compound (CI), $n=1$, and Y is a 3-pyridinyl group.

The compounds of formula (CI) may belong to various families, a first family may be defined as that of the "alkyl ethers", which correspond to the following formula (CII):

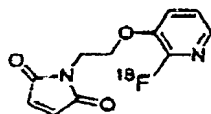


- 15 in which p is an integer from 1 to 10, such as 2, 3, 4, 5, 6, 7, 8 or 9.

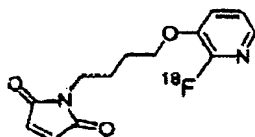
The preferred compounds of formula (CII) are chosen from the following compounds:



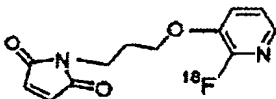
1-[(2-[^{18}F]fluoropyridin-3-yloxy)methyl]pyrrole-2,5-dione



- 25 1-[2-(2-[^{18}F]fluoropyridin-3-yloxy)ethyl]pyrrole-2,5-dione

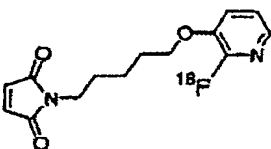


1-[4-(2-[¹⁸F]fluoropyridin-3-yloxy)butyl]pyrrole-2,5-dione

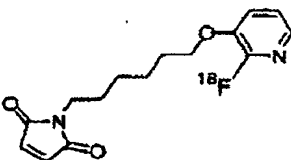


5

1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione



1-[5-(2-[¹⁸F]fluoropyridin-3-yloxy)pentyl]pyrrole-2,5-dione

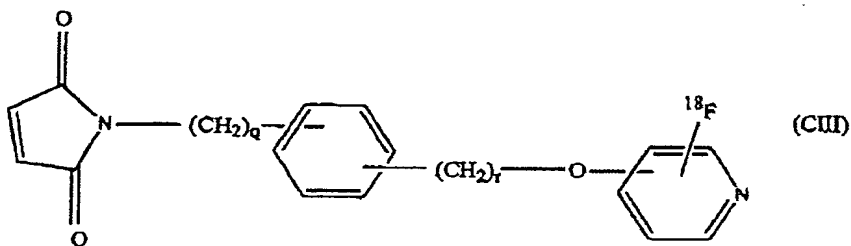


10

1-[6-(2-[¹⁸F]fluoropyridin-3-yloxy)hexyl]pyrrole-2,5-dione

A second family of compounds of formula (CI) may be defined as those of the "phenylalkyl ethers", which correspond to the following formula (CIII):

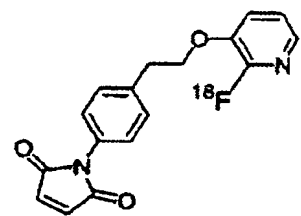
15



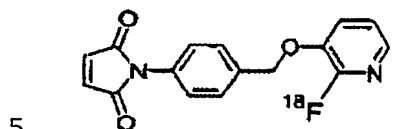
in which q and r represent independently an integer from 0 to 10, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9.

20

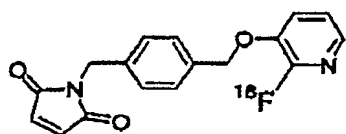
The preferred compounds of formula (CIII) are chosen from the following compounds:



1-{4-[2-(2-[¹⁸F]fluoropyridin-3-yloxy)ethyl]phenyl}pyrrole-2,5-dione



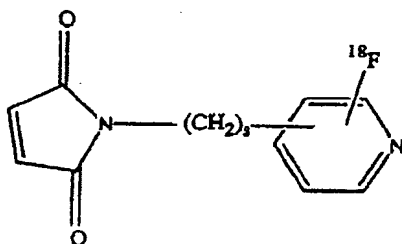
1-[4-(2-[¹⁸F]fluoropyridin-3-yloxymethyl)phenyl]pyrrole-2,5-dione



1-[4-(2-[¹⁸F]fluoropyridin-3-yloxymethyl)benzyl]pyrrole-2,5-dione

10

A third family is that of the compounds which correspond to the following formula (CIV):



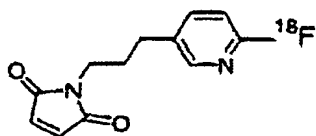
(CIV)

15

in which s is an integer from 1 to 10, such as 2, 3, 4, 5, 6, 7, 8, 9.

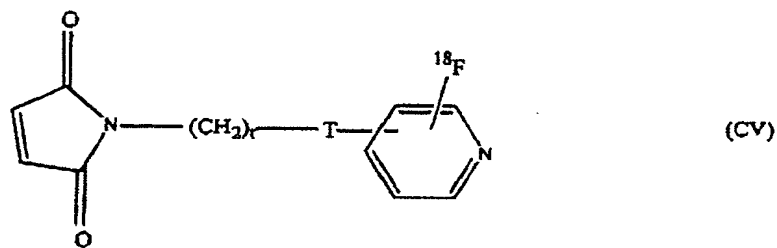
A preferred compound of formula (CIV) is the following compound:

20



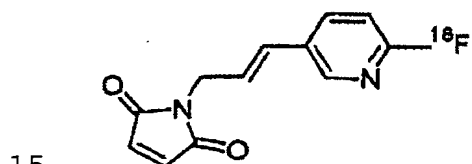
1-[3-(6-[¹⁸F]fluoropyridin-3-yl)propyl]pyrrole-2,5-dione

A fourth family is that of the compounds which correspond to the following formula (CV):

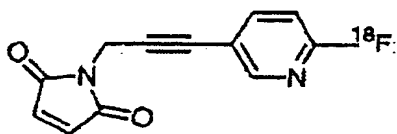


in which t is an integer from 0 to 10, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 and T is a group -CH=CH- or -C≡C-.

Preferred compounds of formula (CV) are the following compounds:



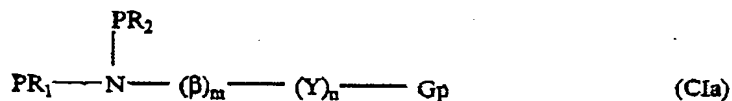
1-[3-(6-[¹⁸F]fluoropyridin-3-yl)allyl]pyrrole-2,5-dione



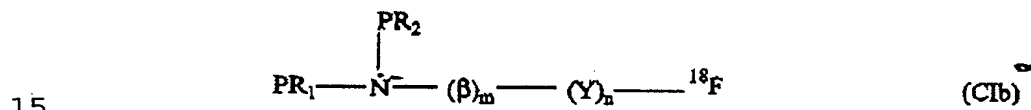
1-[3-(6-[¹⁸F]fluoropyridin-3-yl)prop-2-ynyl]pyrrole-2,5-dione

The labelling compound (CI) may be prepared by a method in which:

a) a precursor compound of formula (CIa):



in which PR_1 and PR_2 represent independently a hydrogen atom or a group protecting the amine functional group, provided that PR_1 and PR_2 are not both (simultaneously) a hydrogen atom, or alternatively PR_1 and PR_2 together form with the nitrogen atom a cyclic group protecting the amine functional group, Gp represents a leaving group which can be replaced with a fluorine-18 atom, and β , γ , m and n have the meanings already given above; is brought into contact with a source of fluoride ions F^- labelled with $[^{18}\text{F}]$, to give a compound of formula (CIb):



b) the group(s) PR_1 and/or PR_2 protecting the amine functional group is (are) removed from the compound (Ib) to give a compound of formula (CIc):



c) the compound (CIc) is reacted with a reagent capable of giving a maleimido group from an amino group, so as to obtain the final compound of formula (CI).

The method according to the invention is simple, reliable, easy to carry out and may be easily automated. It comprises only three steps in which one is an extremely simple deprotection step.

The overall duration of the method is short: by way of example, it is generally from 60 to 120 minutes, preferably from 75 to 85 minutes.

5 The incorporation of the halogen fluorine-18 is carried out in an extremely efficient manner with a high yield, for example 70 to 100%, in particular due to the fact that it is carried out on a heterocyclic group such as pyridine.

10

The final yield of the entire method for a purified product is extremely high, for example from 15% to 25% and the potential quantities of the "synthon" compound, at the end of synthesis, are also very high.

15

In the compound (CIa), the groups PR_1 and PR_2 , when they are protective groups, may be any protective group known in organic chemistry. They are preferably chosen from the tert-butoxycarbonyl (BOC) and fluorenylmethoxycarbonyl (Fmoc) groups.

20

When PR_1 and PR_2 together form with the nitrogen atom of the amine functional group, a group protecting the latter, the protecting group may be for example a phthalimido group.

25

In the compound (CIa), the Gp group may be any leaving group capable of being replaced by a fluorine-18 atom; Gp is preferably chosen from halogens such as F, Cl, Br, I, mesyl, tosyl and triflate groups, when Y is an alkyl group; and Gp is preferably chosen from halogens, ammonium salts, such as trimethylammoniumtrifluoromethanesulphonate, and the nitro group, when Y is an aromatic or heterocyclic group.

30

In step a), the source of fluoride ions labelled with ^{18}F comprises the said fluoride ions and a counter-ion, chosen from large-sized cations such as rubidium, and

tetrabutylammonium, and small-sized cations such as potassium, sodium and lithium, the said small-sized cations being trapped, stabilized, for example by a cryptand or a crown ether, and the like, the said
5 cryptand or crown ether being suitable for the small-sized cation used.

An example of a cryptand is the product KRYPTOFIX[®] K₂₂₂:
(4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexa-
10 cosane) which traps for example the potassium ion.

The counter-ion or cation may be brought in the form of any salt, for example it may be K₂CO₃, in the case of potassium.

15 Step a) is generally carried out in a solvent, which may be any suitable solvent, such as DMSO.

Step a) may be carried out under conditions known to
20 persons skilled in the art, with heating generally at a temperature of 50 to 200°C, for example, 145°C, for a period generally of 1 to 30 minutes, for example of 4 to 6 minutes.

25 Step b) for removing the group protecting the amine functional group, for deprotection, to give the compound of formula (CIc), where the amino group is free, may be carried out by any known deprotection method. It will be possible for example to bring the
30 compound (CIb) into contact with TFA in CH₂Cl₂ for a period generally of 1 to 5, for example of 2 minutes.

It should be noted that TFA is generally used only if the receptor group is removed in an acidic medium, for
35 example when PR₁ = BOC and PR₂ = H.

In step c), the reagent capable of giving a maleimido group from an amido group may be any known compound. It

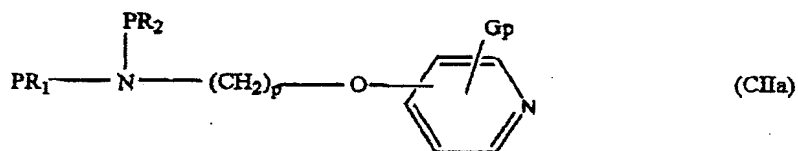
may thus be chosen from N-methoxycarbonylmaleimide and succinimide.

5 Step c) may be carried out under conditions known to persons skilled in the art, for example in a solvent, such as xylene, THF, with heating generally at a temperature of 100 to 200°C, for example of 190°C, for a period of 1 to 20 minutes, for example of 5 minutes.

10 Step c) may, in another embodiment, also be carried out in a biphasic mixture for example of dioxane and aqueous sodium bicarbonate, at room temperature for a period of 3 to 15 minutes, for example 10 minutes; this embodiment of step c) offers the advantage of giving a
15 better yield and of being carried out at room temperature, without the need to heat the mixture.

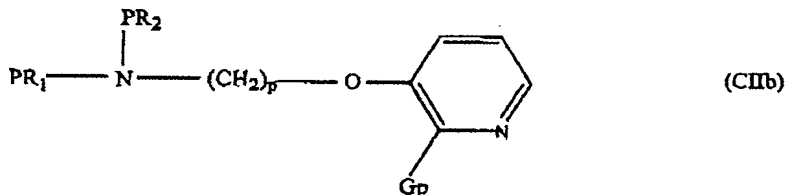
The compound of formula (CIa) may correspond to the following formula (CIIa):

20

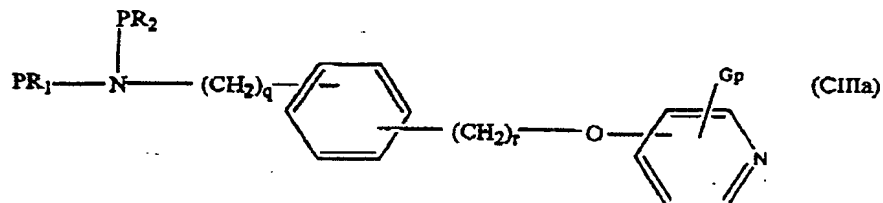


The compound (CIIa) preferably corresponds to the following formula (CIIb):

25

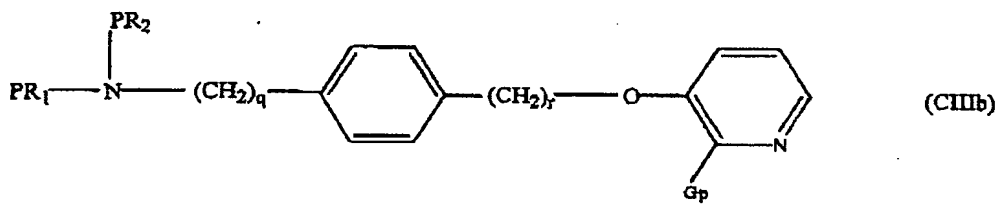


The compound of formula (CIa) may, in another embodiment, correspond to the following formula
30 (CIIIa):



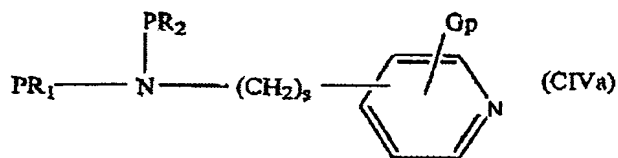
The compound (CIIIa) preferably corresponds to the following formula (CIIIb):

5



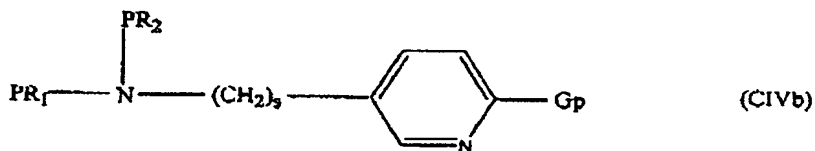
The compound of formula (CIa) may, in yet another embodiment, correspond to the following formula (CIVa):

10



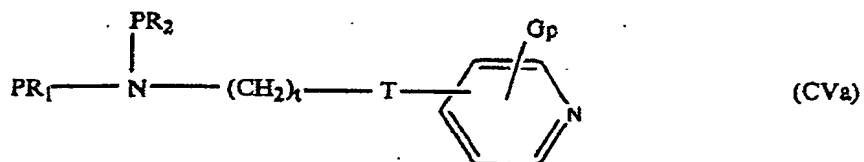
The compound (CIVa) preferably corresponds to the following formula (CIVb):

15

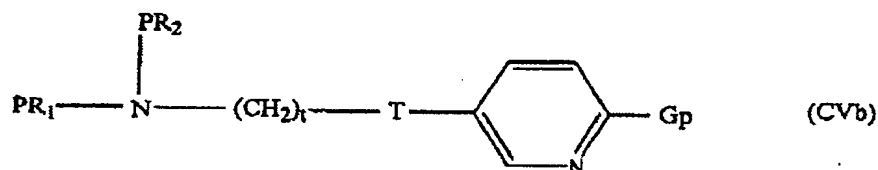


In another embodiment, the compound of formula (CIa) may correspond to the following formula (CVa):

20



The compound (CVa) preferably corresponds to the following formula (CVb):



5

The present invention also relates to a method for synthesizing the peptide labelled with fluorine-18 in accordance with the present invention. This method of synthesis comprises a step of addition of a compound
10 (CI) defined above with a peptide comprising the sequence (PI) defined above. This is indeed an addition reaction carried out between the double bond of the maleimide functional group of the compound (CI) and a free -SH functional group of the peptide, in particular
15 the thiol functional group of a cysteine, of the peptide comprising the peptide sequence (PI). The addition may be carried out directly on a free -SH functional group of the peptide sequence (PI), in particular on the thiol functional group of a cysteine
20 of the peptide sequence, as described above. This addition may be made for example in an acetonitrile/methanol solvent in a ratio of 2:1 by volume, respectively, or in any other appropriate solvent for this type of addition reaction. It will of
25 course be necessary to take care that the solvent used does not affect the peptide (PI) of the invention.

This method therefore has the advantage of being easy to carry out unlike the labelling methods of the prior
30 art.

The coupling will occur, while preserving the activity of the peptide of the present invention, and in general at the ends or at the level of the ends of the peptide
35 of the present invention, on surface residues, or on a

part of the peptide sequence different from the sequence (PI) defined above and in particular on the sequence (PII).

5 The present invention also provides a labelled assemblage having affinity for a phospholipid, comprising at least two peptides comprising the sequence (PI) defined above, which are identical or
10 other, and each or only one of these peptides being labelled by means of a labelling compound (CI) according to the invention. These assemblages may be obtained for example by inserting a flexible peptide linkage, for example polyglycine, between the C-
15 terminal residue of a peptide of the invention and the N-terminal residue of the second peptide and so on depending on the number of peptides joined end to end. This polyglycine linkage may be of formula $-(\text{Gly})_n-$, n being an integer ranging from 1 to 12, for example
20 greater than 4.

These assemblages may also be synthesized by conventional methods of organic chemistry synthesis or protein chemistry, and by genetic recombination *in vivo*
25 or *in vitro*, by genetic engineering, and the like, for example by one of the abovementioned methods.

These assemblages are designed in particular to increase the affinity of the peptides of the present
30 invention for the phospholipid, for example for a negatively charged phospholipid.

A labelled peptide or a labelled assemblage of the present invention may be used in two ways which are
35 research and diagnosis, and there are numerous applications.

The pathologies especially targeted by the present invention are: (i) blood clotting disorders, (ii) the phenomena of apoptosis following the action of chemical compounds, physical effects such as ionizing radiation, biological effects such as those linked to the formation or the necrosis of cancer tissues, in addition to normal apoptosis phenomena, (iii) inflammatory pathologies, and (iv) disorders associated with the relationships between the cells and the extracellular matrix and in particular collagen.

The peptides of the present invention have in addition a great advantage compared with the prior art compounds: the reversibility of their folding processes which allows their handling at high temperatures but which are compatible with the chemical stability of the peptides, for the purposes of chemical modifications with the aim of developing molecules which can be used in imagining.

In addition, because of their small size, the peptides of the present invention may be easily combined with other proteins either to form multifunctional chimeric proteins, or to introduce a mechanism for regulation by effectors other than the signalling phospholipids.

According to the invention, the peptides and assemblages according to the invention coupled to the compound (CI) form labelling compounds which can be used for example for *in vivo* or *in vitro* diagnosis.

Indeed, the peptides of the present invention may be used for the detection of pathologies involving the appearance of negative charges at the surface of cells and the release of microvesicles into the blood: for example clotting disorders, acute inflammatory pathologies and the like, and apoptosis.

The radioactive halogen is fluorine-18 which is a radio element with a short life span because it allows "in vivo" detection of the localization of the thrombotic regions during all sorts of stroke, in particular of the apoptotic and inflammatory foci using appropriate imaging systems.

The peptides or assemblages labelled with fluorine-18, according to the desired application, may be advantageously packaged in the form of diagnostic kits. Thus, the present invention also provides a diagnostic kit comprising a labelled peptide or assemblage in accordance with the present invention.

The present invention also provides a kit for the analysis and detection of negative charges at the surface of cells, characterized in that it comprises a labelled peptide or assemblage of the present invention.

The present invention also provides a kit for the analysis and detection of microvesicles in the blood, characterized in that it comprises a labelled peptide or assemblage in accordance with the present invention.

The peptides labelled with fluorine-18 according to the invention can therefore be used for the manufacture of a product intended for the detection of centres exposing negatively charged lipids at the surface of cells and/or the release of microvesicles into the blood. As specified above, the detection may be a detection by means of scintigraphic images acquired by positron emission tomography, because the compound (CI) comprises ¹⁸F.

In their application, in the context of "PET", the compounds (CI) and the labelled peptides according to the invention, comprising a fluorine-18 atom, show

numerous advantages compared with the compounds with another radioactive halogen, for example iodine.

Indeed, the only positron-emitting iodine isotope is
5 iodine-124, which could allow PET.

However, it is still produced in small quantities (a few mCi against curies for F-18). It is also difficult to produce. Finally, iodine-124 is not a pure positron
10 emitter (fluorine-18, 97%) and decreases by beta+ emission at 25% only and by electron capture at 75%; it possesses a large number of gamma lines ranging from 0.603 MeV (62%) to 2.75 MeV (1%).

15 The invention additionally relates to compositions for analysis and detection for example by positron emission tomography (PET), or compositions for diagnosis comprising a peptide labelled with fluorine-18 as described above and a pharmaceutically acceptable
20 vehicle.

Other advantages and characteristics of the present invention will further emerge on reading the illustrative and nonlimiting examples which follow,
25 with reference to the figures in the annex.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

- The appended sequences ID No. 1 to ID No. 14 are examples of peptides containing the peptide sequences
30 (PI) and (PII) of the present invention.

In particular, the sequences ID No. 11, ID No. 13 and ID No. 14 are examples of peptides containing the peptide sequence of the present invention in which
35 mutations have been introduced in order to increase the affinity for calcium and phospholipids.

BRIEF DESCRIPTION OF THE FIGURES

- Figures 1 and 2 are micrographs obtained from tissue sections of an apoptotic heart (Figure 1) and of a kidney (Figure 2), respectively. These sections were obtained, on the one hand, (photos on the left) with AFIM-fluorescein (AFIM-F) peptides of the present invention, on the other hand (photos on the right) with annexin 5-fluorescein (A5-F) (compound of the prior art): fluorescence microscopy, magnification $\times 40$. The photos in the centre were obtained with haematoxylin: visible light microscopy, magnification $\times 40$. In Figure 1, the top and bottom photos represent different heart sections.

- Figure 3 is a graph which represents the degree of helicity "H" (in %) of a peptide according to the present invention as a function of the temperature "t" in $^{\circ}\text{C}$.

EXAMPLES

Example 1: Synthesis by genetic recombination: expression and purification of the peptides of sequences ID No. 1 to ID No. 12 of the present invention

The sequences ID No. 1 to ID No. 14 were prepared by overexpression in *E. coli* according to the same protocol as that which has been described by F. Cordier-Ochsenbein et al. in J. Mol. Biol. 279, 1177-1185.

The cDNAs of each of these sequences were prepared using a polymerase chain reaction (PCR). They were inserted into the vector pGEX-2T (Smith & Johnson, 1998). Figure 2 is an image illustrating the insertion of the cDNA into the vector. The absence of PCR-induced mutations was checked by sequencing.

The production of the peptides is carried out using the strain *E. coli* BL21 containing the expression vector described above. After induction with isopropylthiogalactopyranoside (IPTG, 100 μ M) to an optical density of 1 to 600 nm, the growth is continued until a plateau is reached, that is to say for about 3 hours. After centrifugation, the bacteria are resuspended in the lysis buffer comprising 50 mM Tris-HCl, pH 8, 10 mM EDTA, 500 mM NaCl, 5% (v/v) glycerol, 1% (v/v) Triton X100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 20 μ g/ml of aprotinin.

The purification was carried out in the following manner: after sonication and centrifugation at 10 000 g, the supernatant containing the soluble proteins is incubated with glutathione/agarose beads allowing specific binding of the GST-domain fusion protein to these beads. After washing with a solution containing 1 M NaCl, 50 mM Tris-HCl at pH 8, 70 units of thrombin per litre of culture are added and the sequences are eluted.

The sequences are then purified on a proRPC (trademark) column of the 16/10 type, supplied by the company Pharmacia using an FPLC system and a linear gradient of water of Millipore (trademark) quality containing 0.1% (v/v) of trifluoroacetic acid TFA, and acetonitrile containing 0.1% of TFA. The flow rate is adjusted to 2.5 ml/minute. The sequences are then freeze-dried.

The final yield for each peptide is about 8 mg of sequence per litre of culture.

Example 2: Example of chemical synthesis of peptides of the present invention

The peptides of the present invention were manufactured in this example by solid phase chemical synthesis with an Applied Biosystems, mod. 433A automatic peptide synthesizer, and by Fmoc chemistry, which uses the
5 fluorenylmethyloxycarbonyl (Fmoc) group for the temporary protection of the α -amino functional group of the amino acids.

The protecting groups used to prevent side reactions of
10 the side chains of amino acids, in this strategy Fmoc, were tert-butyl ether (tBu) for the Ser, Thr and Tyr residues; tert-butyl ester (OtBu) for Asp, Glu; trityl (Trt) for Gln, Asn, Cys, His; tert-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethylchroman-6-
15 sulphonyl (Pmc) for Arg.

The coupling reaction is carried out with an excess of 10 equivalents of amino acids (1 mmol) relative to the resin (0.1 mmol). The protected amino acid is dissolved
20 in 1 ml of N-methylpyrrolidone (NMP) and 1 ml of a 1M solution of 1-N-hydroxy-7-azabenzotriazole (HOAt) in the solvent NMP. 1 ml of a 1M solution of N,N'-dicyclohexylcarbodiimide (DCC) is then added. After 40 to 50 minutes of activation, the active ester formed is
25 transferred into the reactor which contains the resin. Before this step of transfer and then of coupling, the resin is deprotected of its Fmoc group by a 20% solution of piperidine in NMP. The excess piperidine is removed by washing with NMP after about 5 to 10
30 minutes.

During the deprotection, the detection of the dibenzofulvenepiperidine adducts at 305 nm makes it possible to monitor the good progress of the synthesis.
35 Indeed, the quantification of the adduct makes it possible to estimate the efficiency of the deprotection of the Fmoc group and thereby of the coupling of the last amino acid incorporated.

The cleavage of the resin and of the protecting groups present on the side chains was carried out simultaneously by treating the peptide linked to the resin with trifluoroacetic acid (TFA). Before carrying out the cleavage, the resin was washed several times with dichloromethane (DCM) and finally dried. The reagent used during the cleavage is an acid mixture containing 81.5% of TFA and the phenol scavengers (5%), water (5%), ethanedithiol (2.5% when the peptide contains a cysteine) and triisopropylsilane (1%). The resin was treated with this mixture for three hours, with stirring and at room temperature, in an amount of 100 ml of solution per gram of resin. The free peptide in solution was recovered by filtration. The peptide was then precipitated and washed in the cold state in diisopropyl ether and then dissolved in 20% acetic acid and freeze-dried.

The peptide recovered after freeze-drying, the crude material from synthesis, is in reduced form, that is to say that the interchain disulphide bridges are not formed.

The peptide is then purified on a proRPC (trademark) column of the 16/10 type, supplied by the company Pharmacia using an FPLC system and a linear gradient of water of Millipore (trademark) quality containing 0.1% by volume of trifluoroacetic acid TFA, and acetonitrile containing 0.1% of TFA. The flow rate is adjusted to 2.5 ml/minute. The peptide is then freeze-dried.

The products obtained were analysed by mass spectrometry.

Example 3: Stability of the sequences ID No. 1 to ID No. 14

This example shows that the peptides of the present invention constitute stable folding proteins.

Composition of the blank (control):

5 Tris 50 mM, NaCl 150 mM, DTT 1 mM pH 8 10 μ l
H₂O 990 μ l
Adjusted to pH 8

Composition of the sample:

10 Sample: domain purified in 50 mM Tris buffer containing
150 mM NaCl, pH 8 Approx. concentration: 200 mg.ml.
Domain: 10 μ l that is 300 μ M final.
H₂O: 990 μ l
pH measured at 7.8.

15

Hardware and software configuration:

Apparatus Jobin Yvon CD6.
Software CD-max
Optical path length of the measurement cuvette: 1 cm.

20

The appended Figure 1 represents the degree of helicity of AFIM as a function of the temperature as measured with the aid of the circular dichroism signal in far UV at the wavelength of 220 nm.

25

In this figure, the value of the signal at 14°C is taken as 100% of the helical content of the peptide. Heat denaturation of the peptide is indeed cooperative and demonstrates that at low temperature and in particular at 37°C, this is a peptide which is suitably folded and exhibiting enhanced stability.

30

Example 4: Assemblages of two peptides of the present invention

35

The method described in Example 1 above is used to synthesize a peptide sequence with the sequence ID No. 1-(gly)₄-ID No. 1.

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The final yield for the assemblage is about 14 mg/litre of culture.

5 This assemblage may be labelled with a radioactive halogen according to the present invention, in the same manner as the peptide alone, for example by the method described below.

10 Example 5: Synthesis of a labelling compound of the present invention

In this example, there is described the preparation of a labelling compound according to the invention, which
15 is 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione.

a) Complex K[¹⁸F]F-K₂₂₂.

20 In order to recover and recycle the water target [¹⁸O], it is caused to pass through an anion-exchange resin (AG1x8, from Bio-Rad, 100-200 mesh). The fluoride [¹⁸F] ion is then eluted from the resin, using 1.0 ml of an aqueous solution of K₂CO₃ at 4.5 mg/ml.

25 After addition of 11.0 to 15.0 mg of KRYPTOFIX[®] K₂₂₂ (4,7,13,16,21,24-hexaoxa-1,10-diazobicyclo[8.8.8]hexacosane), the resulting solution is then gently concentrated to dryness at 145-150°C, under a nitrogen
30 stream for 10 minutes in order to give a pure K[¹⁸F]F-K₂₂₂ complex, in the form of a white semisolid residue.

b) 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-
35 2,5-dione

Freshly distilled DMSO (600 µl), containing 4.0 to 6.0 mg of the "nitro" marker precursor (tert-butyl
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ester of [3-(2-nitropyridin-3-yloxy)propyl]carbamic acid) is added directly to the tube containing the dried $K[^{18}F]-K_{222}$ complex. The tube (not sealed) is then placed in a heating block (at 145°C for 4 minutes). The
5 tube is then cooled using an ice/water bath and the remaining radioactivity is measured.

85% to 95% of the initial activity placed in the container is still present. The reaction mixture
10 obtained, which is dark in colour, is then analysed by radiochromatography. The incorporation yields are calculated from the radiochromatogram by TLC and are defined by the ratio of the surface of the tert-butyl ester of [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid to the total activity of the
15 ^{18}F fluorine-18 (SiO_2 -TLC; eluent: EtOAc; Rf: 0.75 and Rf: fluoride [^{18}F] ion: 0.0). The reaction mixture is diluted with 1 ml of water and transferred into a C18 Sep-pak cartridge (Waters). The tube is rinsed
20 twice with 1 ml of water, which is also transferred and added to the dilute reaction mixture in the cartridge.

The whole is then caused to pass through the cartridge. The cartridge is washed with 3 ml of water and
25 partially dried for 0.5 minute, by sending a nitrogen stream.

The tert-butyl ester derivative of [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid is eluted from the
30 cartridge with 3 ml of dichloromethane in a reaction flask containing 0.1 ml of TFA. Twice 1 ml of dichloromethane are used to wash the cartridge and to completely transfer the [^{18}F]-labelled derivative mentioned above (5% of the total quantity of
35 radioactivity involved in the fluorination process remains on the cartridge). The incorporation yield is also confirmed after elution of the Sep-pak by the ratio of the count values for CH_2Cl_2 to the total

radioactivity eluted (DMSO/H₂O+CH₂Cl₂). The resulting CH₂Cl₂/TFA solution (50/1, V/V) is concentrated to dryness (at 65-75°C) under a moderate nitrogen stream for 4 to 6 minutes). The deprotection yield is
5 quantitative: no molecule described above, protected with BOC, can be detected by radiochromatography. The above residue is redissolved in 2 ml of CH₂Cl₂ and again concentrated to dryness in order to minimize the presence of TFA (at 65-75°C under a moderate nitrogen
10 stream for 4 to 6 minutes). The residue is then diluted with 0.5 ml of xylene containing 25 mg of N-methoxycarbonylmaleimide. The container is then hermetically closed, heated for 5 minutes at 190°C (strong reflux), and then cooled for 2 minutes, using
15 an ice-water bath. The reaction mixture is then injected onto a semipreparative HPLC column. Isocratic elution [eluent: heptane/EtOAc: 50/50; flow rate: 6.0 ml/minute] which gives pure labelled 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione,
20 retention time: 7.5 to 8.0 minutes.

Typically, 60 to 70 mCi of pure labelled 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione may be obtained in 75 to 85 minutes, from 550-650 mCi from
25 an [¹⁸F]F⁻ production batch of a cyclotron.

Example 5a:

The compound labelled with fluorine-18, 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione may
30 also be prepared by repeating steps a) and b) of the method described in Example 5, still using, as labelling precursor, the "nitro" compound (tert-butyl ester of [3-(2-nitropyridin-3-yloxy)propyl]carbamic
35 acid), but modifying the final part of the preparation (step c)) in the following manner (variant according to which step c) is carried out in a biphasic mixture of dioxane and aqueous sodium bicarbonate).

After deprotection of the amine functional group (TFA/CH₂Cl₂), the residue obtained after concentration to dryness is taken up in 0.250 ml of dioxane
5 containing 25 mg of N-methoxycarbonylmaleimide. To this solution, 0.750 ml of a saturated aqueous sodium bicarbonate solution is added, and the preparation is vortexed at room temperature for 10 minutes. The reaction mixture is then diluted with 1 ml of water and
10 transferred onto a C18 Sep-pak cartridge (Waters). The flask is rinsed twice with 1 ml of water, which is also transferred and added to the dilute reaction mixture in the cartridge. Finally, 8 ml of water are again added to the dilute reaction mixture in the cartridge. The
15 whole is then passed through the cartridge. The cartridge is washed with 3 ml of water and partially dried for 0.5 minutes, by sending a nitrogen stream. The derivative labelled with fluorine-18 (1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione) is
20 eluted from the cartridge with 3 ml of dichloromethane in a new empty flask. 1 ml of dichloromethane is used twice to wash the cartridge and to completely transfer the [¹⁸F]-labelled derivative mentioned above. The solution containing the abovementioned [¹⁸F]-labelled
25 derivative is concentrated (at 65-75°C, under a moderate nitrogen stream for 3 to 5 minutes) to a volume of about 1 ml and injected onto a semipreparative HPLC column. The purification is identical to that described in Example 5.

30

Example 5b:

The compound labelled with fluorine-18, 1-[3-(2-[¹⁸F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione may
35 also be prepared by repeating steps a) and b) of the method described in Example 5 or 5 b, but using, as labelling precursor, the compound "trimethylammonium trifluoromethanesulphonate" ([3-(3-tert-butoxycarbonyl-

aminopropoxy)pyridine-2-yl]trimethylammonium trifluoromethanesulphonate).

Example 6: Labelling of a peptide of the present invention with fluorescein

This example, as well as Example 7 which follows, are intended to demonstrate the efficiency of recognition of apoptotic sites by the peptides of the present invention.

In the examples which follow, the peptide of the present invention is called AFIM-SH. It has a peptide sequence as defined by the sequence (PI). The sequences ID No. 1 to ID No. 14 are tested.

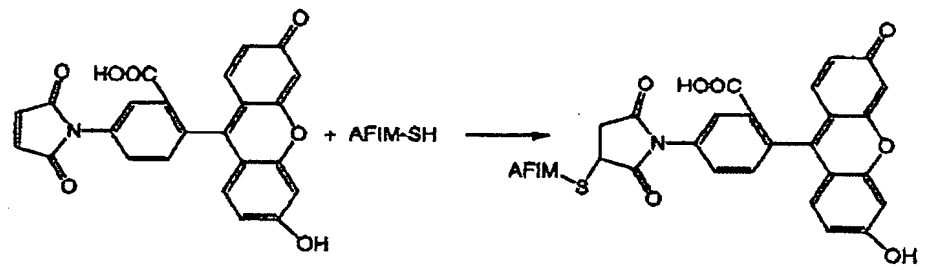
Fluorescein is a molecule which emits a green fluorescence having a wavelength of 525 nm when it is excited at a wavelength of 488 nm. The emission of green light is detected by cameras or photomultipliers. This coupling of AFIM to fluorescein makes it possible to detect the presence of the cells exhibiting PS both *in vitro* and *in vivo* in small animals.

According to the present invention, it is possible to label AFIM at the level of the surface residues on any cystein which would be introduced in place of any amino acid present at the surface of AFIM (surface residues) as long as the function for binding to the lipid membranes is not disrupted. AFIM thus modified is designated AFIM-SH below.

The coupling of fluorescein occurs via a maleimide functional group represented below on AFIM by the SH functional group.

The fluorescein is covalently coupled to one or more cysteines of the sequence using a maleimide functional group.

5 General labelling scheme (scheme I):



10 The entire labelling is performed at a temperature of less than 20°C.

AFIM-SH is in solution in Tris buffer, (50 mM), containing NaCl (150 mM), pH = 7.4. 5 equivalents of DTT in solution in the same buffer are added to the AFIM-SH solution. The medium is stirred for 30 min.

Protected from light: the fluorescein (5 equivalents of AFIM-SH + 2 equivalents of DTT) is weighed and dissolved in DMF, and added to the preceding solution. The whole is stirred, and the reaction is continued for 30 min. Next, the medium is diluted in 150 ml of PBS buffer (20 mM phosphate, 150 mM NaCl), pH = 7.4, and ultrafiltered on YM3 (trademark) membrane. The sample is rediluted and ultrafiltered several times, determining the UV spectrum of the filtrate.

When there is no longer any fluorescein in the filtrate (peak at 490 nm), the sample is concentrated to a few ml and stored in the cold at 4°C.

30 The AFIM-fluorescein products were used to detect apoptotic cells by flow cytometry *in vitro*, and in

animals in vivo in the manner described in Example 7 which follows.

Example 7: Results for labellings of apoptotic cells
5 with the AFIM-fluorescein products of Example 6

Imaging of apoptotic cardiac cells following a heart attack in rats.

10 A model of apoptosis in rats is used as described in the article which appeared in *Circulation Res.* 1996, 79, 946-956.

Briefly, four rats (300 g each) were anaesthetized,
15 intubated and ventilated. Myocardial ischaemia was triggered by a transient occlusion of the coronary artery. After 30 minutes of occlusion, the coronary artery was reperfused for one hour.

20 At the end of the reperfusion period, the AFIM-fluorescein peptides of Example 6 were injected into the jugular vein in an amount of 200 µg of peptide for each of two of the rats in a total volume of 1 ml.

25 By way of comparison, 200 µg of annexin 5-fluorescein (compounds of the prior art) were injected under the same conditions for each of the other two rats in a total volume of 1 ml.

30 The rats were sacrificed after 60 minutes.

Five organs were stored for this study: the heart, the lung, the kidney, the liver and the brain. They were washed and rinsed in the presence of formalin. The
35 organs were then dehydrated and impregnated with paraffin for about 12 hours and then 7 µm sections were prepared.

A few sections were stained with haematoxylin. The sections were examined under a fluorescence microscope and the adjacent sections stained with haematoxylin were examined with a visible light microscope. The
5 sections stained with haematoxylin (labelled H1 and H2 respectively in the appended Figures 1 and 2) allow visualisation of the architecture of the tissues and fluorescence microscopy to detect the labelling with AFIM-fluorescein (AFIM-F) or with annexin 5-fluorescein
10 (A5-F).

The appended Figure 1 shows the images obtained for the apoptotic heart and the appended Figure 2 shows the images obtained for the kidney.

15 Figure 1 clearly shows the excess of fluorescence corresponding to the accumulation of marker at the level of the apoptotic cells. The contrast is visibly better with AFIM of the present invention than with the
20 prior art annexin 5.

Figure 2 shows the labelling of the kidney linked to the partial elimination of the products. In the case of AFIM, the glomeruli do not appear to be labelled, only
25 the proximal tubules are partially labelled. On the other hand, in the case of the prior art annexin 5, the entire renal tissue is strongly labelled, which is in agreement with the renal toxicity observed for this protein.

30 The results obtained in this example demonstrate a high specificity of the peptides of the present invention for the labelling of the cells.

35 The labelling of the AFIM peptide, for example from ID No. 1 to 10, by fluorescein therefore makes it possible to efficiently detect the phosphatidylserine (PS) present at the outer surface of the cells involved in

physiopathological processes such as programmed cell death (apoptosis), blood clotting, inflammatory reaction.

- 5 **Example 8:** Labelling according to the method of the present invention of peptides comprising the sequence (PII) with the labelling compound (CI)

10 In the examples which follow, the peptide of the present invention is called AFIM-SH. It has a peptide sequence as defined by the sequence (PII). The sequences ID No. 1 to ID No. 14 of the appended sequence listing are tested. The labelling compound called synthon ^{18}F manufactured in Example 5 (or 5 a or 15 5 b) is used in this example.

AFIM is coupled, specifically at the level of an SH functional group of the cystein J^{\square} to the synthon ^{18}F .

- 20 The general scheme for the labelling may be summarized in the following manner:



- 25 AFIM-SH is in solution in Tris buffer (50 mM) containing NaCl (150 mM), pH = 7.4. The synthon ^{18}F is dissolved in an acetonitrile-methanol (2/1 v/v) mixture, and AFIM-SH is added. The whole is stirred, and the reaction is continued for 3 minutes at room 30 temperature.

The reaction medium is then transferred onto a column of maleimide beads suspended in DMF, and eluted with PBS buffer.

- 5 The medium is purified by HPLC on an exclusion gel column, and eluted in PBS buffer (20 mM KH_2PO_4 , 150 mM NaCl, pH = 7.4).

10 The product, once purified is intravenously injected into rats.